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Physical and Biological Studies on Transforming DNA

A. T. GANESAN AND J. LEDERBERG

Department of Genetics, Stanford University School of Medicine
Palo Alto, California, U.S.A.

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Bacillus subtilis transforming DNA prepared by the method described, has an average molecular weight of 11·6 million. The DNA is heterogeneous, as judged by chemical composition, thermo-spectral and pycnographic properties. These properties have been used to fractionate some of the biologically active molecules which show higher specific activities in transforming respective auxotrophs.

1. Introduction

The DNA-mediated transformation in *Bacillus subtilis* (Spizizen, 1958; Nester & Lederberg, 1961), offers a favorable opportunity to study the physical and chemical properties of DNA molecules in relation to their biological activity. The average guanine–cytosine content in the DNA varies widely from one bacterial species to another. It is relatively uniform for the molecules within each bacterial species; the coefficient of standard deviation (d) in guanine–cytosine content (G–C) is less than 0·03 (Rolfe & Meselson, 1959; Sueoka, Marmur & Doty, 1959; Sueoka, 1960). The G–C ratio can be estimated pycnometrically and also from the thermospectral measurement of transition from a bihelix to random coil as well as by direct analysis. These effects can be used to separate and analyse DNA fractions and relate their compositions to their biological properties.

2. Materials and Methods

(a) *Strains*

The mutants here are produced by ultraviolet irradiation of cells or nitrous acid treatment of DNA (Nester & Lederberg, 1961).

List of strains	Genotype	Growth response
SB5	$Try_2\ His_1\ Ura_1$	Tryptophan, histidine, uracil
SB26	$Try_2\ Met_3$	Tryptophan, methionine
SB58	$Try_2\ Ade_1$	Tryptophan, adenine
SB71	His_4	Histidine
SB81	$Try_2\ His_5$	Tryptophan, histidine
SB95	$Try_2\ Arg_7$	Tryptophan, arginine
SB133	Phe_1	Phenylalanine
SB202	$Try_2\ Aro_2\ His_2\ Tyr_1$	Tryptophan, shikimic acid, histidine, tyrosine
SB287	$Try_2\ His_{12}$	Tryptophan, histidine
SB300	$Aro_3\ Tyr_2\ His_{13}$	Histidine, shikimic acid, tyrosine
SB400	His_{22}	Histidine

(b) *Preparation of DNA (see also Nester & Lederberg, 1961; Nester, Ganesan & Lederberg, 1963)*

DNA was prepared by lysing the cells with lysozyme and precipitating with cold ethanol, followed by extraction with 2 M-NaCl + 0·05 M-sodium citrate. The resulting viscous solution was deproteinized with chloroform–octanol, treated with RNase, then twice with Norite to remove nucleotide fragments. Finally, trypsin was added to the DNA solution and followed by another deproteinization. The resulting solution was dialysed for 48 hr against standard saline citrate (0·15 M-NaCl–0·015 M-sodium citrate, adjusted to pH 7). The DNA was precipitated in cold ethanol and the resulting fibrous precipitate dissolved in the required solvent, usually 2 M-NaCl–0·015 M-sodium citrate. Such a purified DNA had less than 1% contaminating protein and less than 3% RNA. The mean density of the DNA in CsCl solution was 1·703 g/cm^3. At 1·5 μg/ml. (a saturating level), it had a transforming efficiency of up to 1% and an average molecular weight of 11·6 million. (This value is calculated from the bandwidth in CsCl, and is without the empirical correction of 2 suggested by Thomas & Berns (1961).

The transformation assays follow the methods of previous publications and have been fully recorded by Ganesan (1963). The strain SB5 requires an addition of 50 μg/ml. of uracil in CH T_1 and 5 μg/ml. in CH–T_2 medium for proper transformation.

(c) *Pycnography (density-gradient centrifugation)*

Cesium chloride (American Potash, Trona) was purified so that solutions of 55% w/w had absorbancies of less than 0·02 at 260 mμ (Kaiser & Hogness, 1960). For centrifugation (Meselson, Stahl & Vinograd, 1957), 35 to 45 μg of purified DNA was added to 2·8 ml. cesium chloride, in 0·01 M-tris buffer, pH 8·5, plus 0·001 M-EDTA. In experiments with partially denatured DNA, 90 to 100 μg were added to enable recovery of assayable amounts of a native, thermally resistant fraction. The final density was adjusted to 1·700 g/cm^3 in a volume of 3 ml. The sample was placed in the tubes of the SW39L head under 2 ml. of purified mineral oil, and spun in the Spinco model L centrifuge at 30,000 rev./min for 82 to 85 hr at 4°C.

The sedimented samples were fractionated by puncturing the tube with a needle and collecting 2 or 3 drops per fraction (one drop = 28 $\pm$ 4 μl.). The recovery (from the gradient) was 80 to 90% of total input DNA. The density of these collected fractions was estimated from the refractive index, n_d, of the solution (Weigle, Meselson & Paigen, 1959). For the final density determination, these fractions were sedimented in the model E analytical centrifuge in a cell containing a Kel-F centerpiece at 44,770 rev./min, at 25°C (Sueoka, 1961). 1 to 2 μg of a *Micrococcus lysodeikticus* DNA the density of which in CsCl is 1·73 g/cm^3 was added as a standard for accurate calibration (Rolfe & Meselson, 1959).

(d) *Assay*

After reading the optical density at 260 mμ, the fractions were diluted to give a concentration of 0·001 O.D. units/ml., which when added to 0·9 ml. of medium containing competent cells, became 0·0001 O.D. units. (1 O.D. unit = 47 μg = 141 mμmoles of DNA/ml.). This concentration was in the linear range of the dose–response curve. As a control, similarly collected fractions were mixed and the mixture was diluted to the same concentration. The transformation procedures were performed as described by Nester & Lederberg (1961). At this dilution, the CsCl did not have any inhibitory effect on transformation.

(e) *Thermal denaturation*

In all experiments, standard saline citrate was used as a solvent, and the DNA was dissolved in it at a concentration of 20 μg/ml. The absorbancy of solutions was measured with a Zeiss PMQ11 spectrophotometer equipped with thermostatically controlled housings (Inman & Baldwin, 1962). DNA solutions were maintained at a given temperature, as indicated by the calibrated thermistor, for 15 min and samples were removed with a warmed pipette and cooled rapidly by diluting in chilled solvent (0°C) to prevent renatura-

tion and nonspecific aggregation. To study the heated DNA, the fully denatured component was removed by treatment with an *Escherichia coli* phosphodiesterase (kindly given by Dr Lehman), an enzyme specific for the single-stranded DNA (Lehman, 1960). The reaction was performed according to the procedures described by Lehman (1960), and terminated by chilling and adding NaCl to 0·15 M, a concentration which inhibits the enzyme.

(f) *Renaturation*

Denatured DNA collected from the gradient was dialysed against 0·3 M-NaCl–0·03 M-sodium citrate, renatured, and assayed at 0·01 to 0·001 O.D. units. (The renatured DNA has only 40 to 50% of the initial activity.) The denatured DNA was prepared by heating a solution (O.D. at 260 mμ of 0·60 to 0·65) in standard saline citrate at 100°C for 15 min in a glass-stoppered tube in an ethylene glycol bath, followed by rapid cooling. After 4 hr, its hyperchromicity was only 15%, although during heating this reaches 35 to 40%. This DNA had no biological activity, formed a band at a heavier stratum, 1·718 to 1·720 g/cm^3 than the undenatured material, and was highly susceptible to *E. coli* phosphodiesterase. Three ml. of such a DNA in 0·3 M-NaCl–0·03 M-sodium citrate was put in the thermostatically controlled spectrophotometer cuvette and heated in steps of 5 min duration at 26°, 37°, 50°, 60° and 67°C, when there is a hyperchromicity of 25 to 30%. At 67°C, the absorbance starts to decrease slowly and returns to the initial level within 55 min. This DNA was cooled at room temperature for 2 hr and then stored at 0°C. The procedure gave a renaturation efficiency for transforming activity of 45 to 55% (Marmur & Doty, 1961).

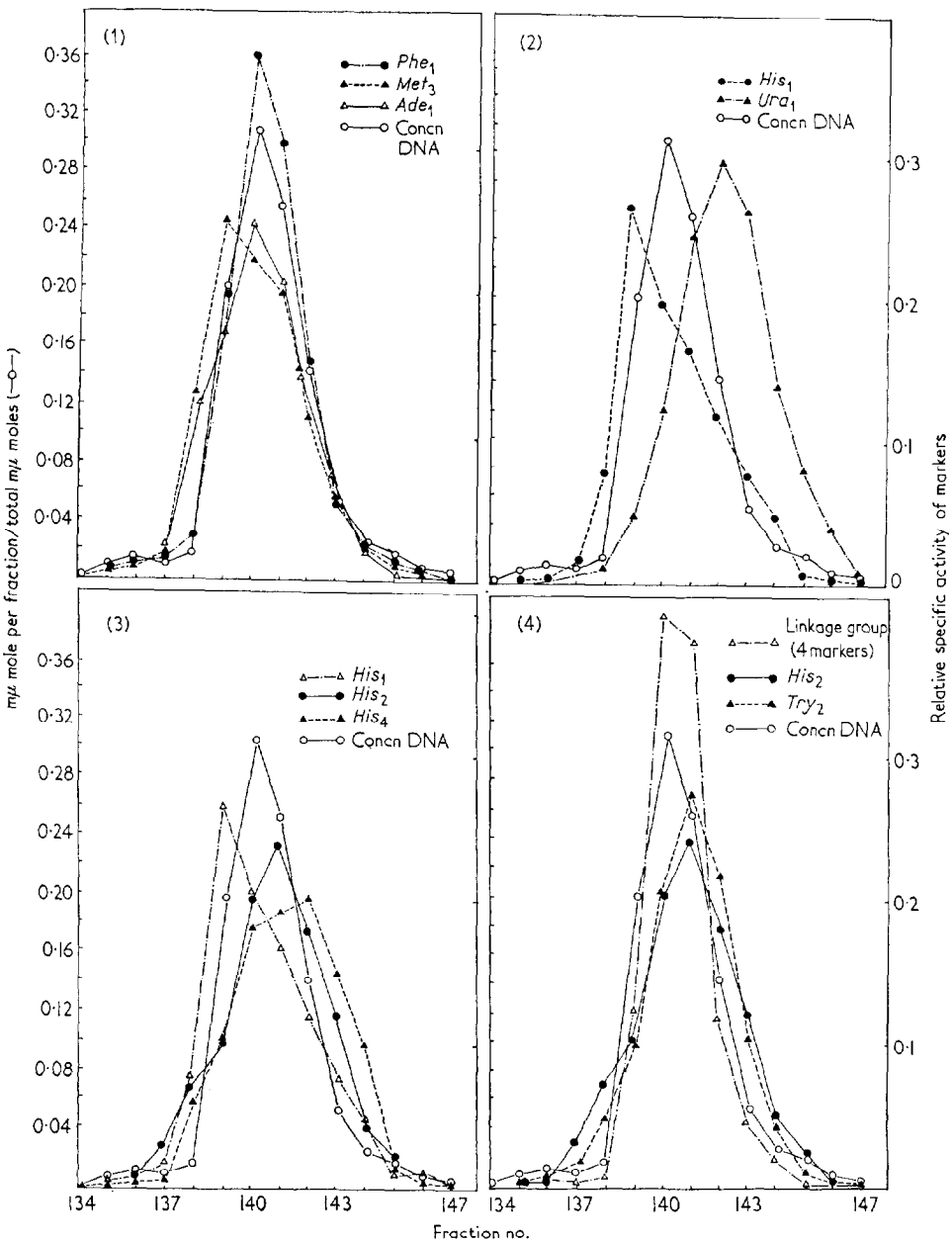
(g) *Base analysis of thermally resistant fraction*

Base compositions were determined by paper chromatography. Purified [^{32}P]DNA (Lehman, 1960) having a specific activity of 1×10^5 cts/min/mμmole was heated to 93·2°C and run in the preparative gradient to separate denser, denatured DNA molecules from a residual undenatured fraction. This thermally resistant fraction at the 1·706 stratum was treated with *E. coli* phosphodiesterase to remove the denatured molecules of DNA. The resulting native DNA was again denatured and treated with the enzyme to convert it into mononucleotides. A comparable amount of control DNA was also treated as above but without centrifugation. Both of these were spotted, along with four mononucleotides as markers, on a Whatman no. 1 filter paper using the isobutyrate–ammonia solvent system (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950). After running for 30 hr, the spots were cut out, eluted and their radioactivity counted. An alternative, less precise method of spectral analysis (Fredericq, Oth & Fontaine, 1961) was especially useful for analysing unlabeled DNA. The enzyme digest was acidified to pH 3·0 and absorbance ratios taken at 260 mμ : 280 mμ to estimate the base composition. The amounts of enzyme added were of no consequence for the optical measurement.

3. Results

The results presented here are from two DNA preparations, one labeled with ^{32}P and the other without any label. Both have an average molecular weight in the range of 10 to 12 million. The distribution of transforming activity for a number of different genetic markers was examined following pycnographic separation. Those which showed reproducibly different distribution patterns and considerable increase in their specific activities, compared to the control, were analysed more intensively. These genetic markers showed thermal transition profiles in fairly good agreement with those predicted from pycnography. The distribution of biological activity for different markers in different fractions is presented in Table 1 with that of control values.

Linkage represents the ability of the fraction to transform all four markers, Aro_2, Try_2, His_2, Tyr_1. Figures 1, 2, 3 and 4 represent the specific activities plotted as probability distributions against fraction numbers. For comparison, these graphs also



FIGS 1 to 4. Distribution of biological activity for the genetic markers in the CsCl density-gradient. The methods are described in the text. The specific activities are plotted as probability distributions.

show the observed span of DNA distribution in the gradient. The density distributions of DNA molecules range from 1·701 to 1·706. In the statistical analysis of fractionation, it was assumed that there were no errors in plating, or determination of DNA concentration and dilutions, so that the colony counts followed a Poisson distribution. Hetero-

TABLE 1

Specific activities of DNA fractions
Transformants per fraction, 0·3 picomole (180×10^9 nucleotide pairs) DNA

Fraction no.	His_1	Ura_1	Try_2	Ade_1	Met_1	Phe_1	His_4	Arg_7	His_2	*Aro* linkage group
136	0	0	0	0	0	0	0	0	0	0
137	6·50	0	3	3	2·0	6	1·5	0	19·5	0
138	30·0	5·5	18	24·5	12·0	12·5	20·0	2·5	22·0	0·5
139	99·0	9·5	48·5	36	22·5	73·5	24·0	7·0	65·0	15·0
140	80·0	21·0	92·5	50·5	20·5	137	37·0	10·5	123·0	30·0
141	58·5	43·0	119	47·5	17·5	113·5	41·0	18·5	152·0	47·5
142	48·0	52·5	94	29·5	10·5	43·0	43·0	23·5	110·0	20·0
143	33·5	29·0	40·5	16·0	4·5	12·5	32·0	18·5	73·0	5·5
144	19·0	16·5	18·5	5·5	1	7·5	15·0	10·5	40·0	3·0
145	2·0	2·5	2·5	0·5	1·5	1·0	3·0	2	25	0
146	0	0	0·5	0·5	0·5	0	0	0	1	0
Pooled control	35	12·6	34·3	28	15·3	75·3	54	10	69·5	2

geneity χ^2 for 10 degrees of freedom comparing transforming activity with distribution of O.D.$_{260\,m\mu}$ ranged from 78·9 for Arg_7 to 624·80 for Try_2.

It can be seen that even if the experimental variance is four times that expected from a Poisson distribution, all the fractionations are significant at the 1% level.

An appreciable proportion of the significant fractionation indicated by these χ^2 values is due to the narrower distributions of genetic activity as compared to the optical density at 260 mμ. This probably reflects the higher proportion of small DNA molecules with lower genetic activity in the tails of the DNA size distribution. Pairwise χ^2 analyses were made for different markers to analyse the extent of fractionation (Table 2). The His_2 marker distribution is wider than that of Try_2. Molecules capable

TABLE 2

Pairwise χ^2 analysis for some markers in the fractionation

Comparison	χ^2_{10}	Comparison	χ^2_{10}
His_1 : Ura_1	149·27	Try_2 : Linkage group	28·67
His_1 : Try_2	120·54	Try_2 : His_2	82·62
His_1 : Phe_1	122·24	Met_3 : Ade_1	11·21
His_1 : His_4	60·74	His_2 : Linkage group	70·03

of transforming all the linked markers show a narrow distribution. For the His_2 marker, significant deviations from the Try_2 distribution occur in both tails. Pairwise analysis of Try_2 *versus* linkage is less significant. One can conclude that the smaller molecules are in relative excess in both tails of the His_2 distribution. The Phe_1, Met_3 and Ade_1 markers show similar distribution and behavior. The striking difference is the distribution of histidine markers, reflecting heterogeneity either in their composi-

tion or in the molecules in which they are located. The plot of the ratio of specific activities for His_1 to Ura_1, Ura_1 to His_1, and Phe_1 to Ura_1, against respective fraction number, is represented in Fig. 5.

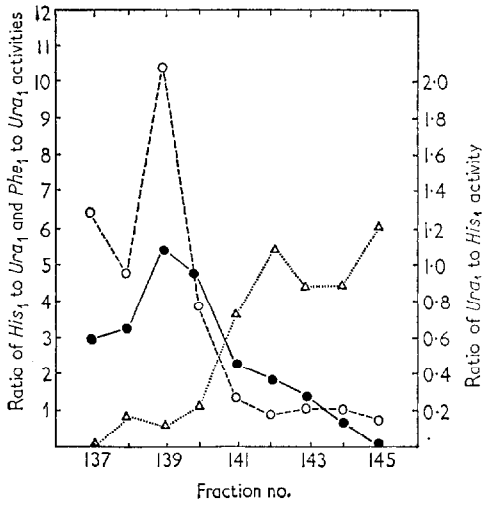
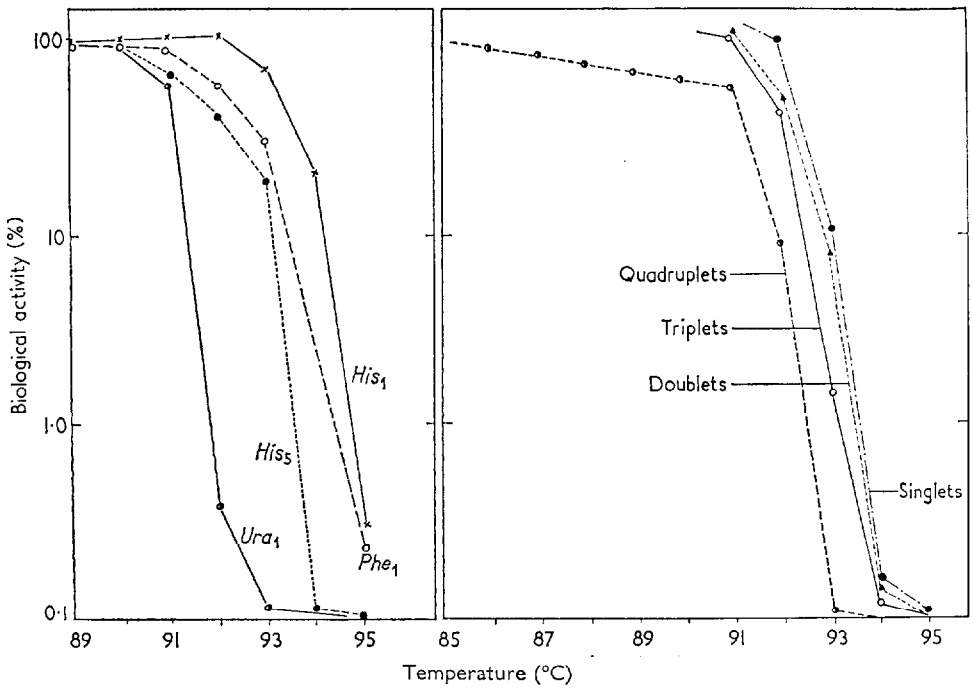


FIG. 5. Plot of the ratio of the specific activities for His_1 : Ura_1, --○--○--; Phe_1 : Ura_1, —●—●—; and Ura_1 : His_1, ...△...△....



FIGS 6 and 7. Thermal denaturation profiles of *B. subtilis* DNA in standard saline citrate as judged by the loss in transforming activity for linked and unlinked markers. The T_m as judged by hyperchromicity was 87·8°C. Linkage was assayed using SB202 as the recipient and scoring for cotransfer of markers, following Nester *et al.* (1963).

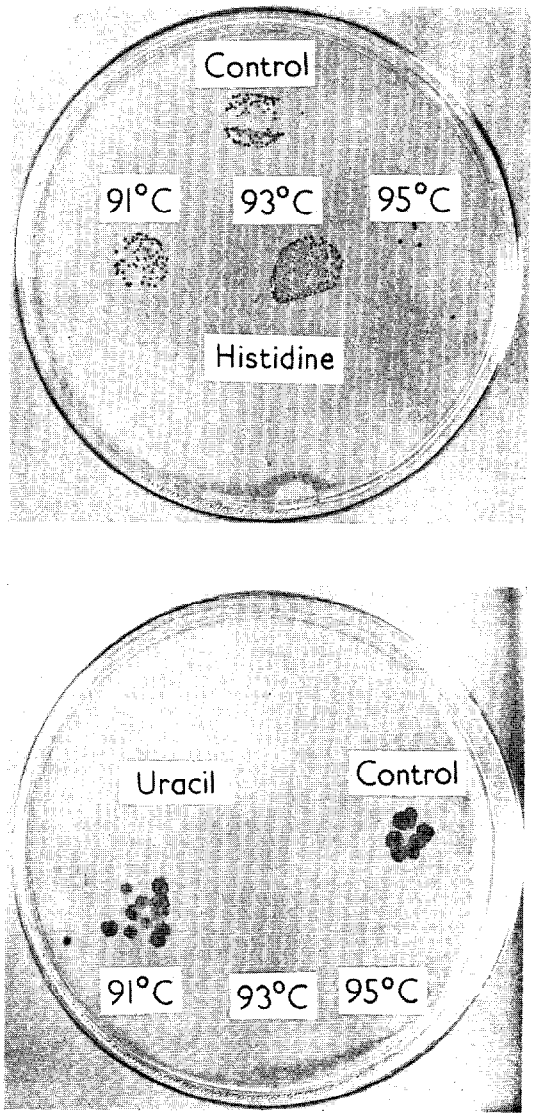


PLATE I. Qualitative spot tests for the loss in transforming activity on heating. 0·01 ml. of control DNA (20 μg/ml.) in standard saline citrate was spotted along with DNA heated at 91°, 93° and 95°C on plates containing minimal medium with necessary supplements. Each plate was spread with 10^7 competent cells carrying His_1 or Ura_1 marker. Transformants appeared after 16 hr of incubation at 37°C.

[*facing p. 689*

TABLE 3

Physical properties of genetic markers

Marker	T_m(B)† ± 0·2 (°C)	Density range‡ (g/cm³ ± 0·002)
Arg$_1$	91·8	1·702
His$_1$	93·8	1·706
His$_4$	92·5	1·703
Met$_3$	92·8	1·704
Phe$_1$	92·8	1·704–1·706
Ade$_1$	92·8	1·704–1·706
His$_5$	91·8	1·703–1·706
Ura$_1$	91·4	1·701–1·703
Linked Markers:		
Try$_2$	92·5	1·702–1·703
His$_2$	92·5	1·702
Aro$_2$	92·5	1·702
Tyr$_1$	91·5–92·5	1·702
Pooled values for linkage group:		
Singlets	92·5	1·702
Doublets	91·8	1·702
Triplets	91·4	1·702
Quadruplets	91·0	1·702

† T_m(B), (marker inactivation), midpoint at which 50% of the biological activity is lost as judged by transformation.

‡ Calculated from the slope of the gradient.

The efficiency of the purified fractions in transforming a particular marker was of the order of 2·5- to 4-fold that of control DNA, depending on the marker assayed.

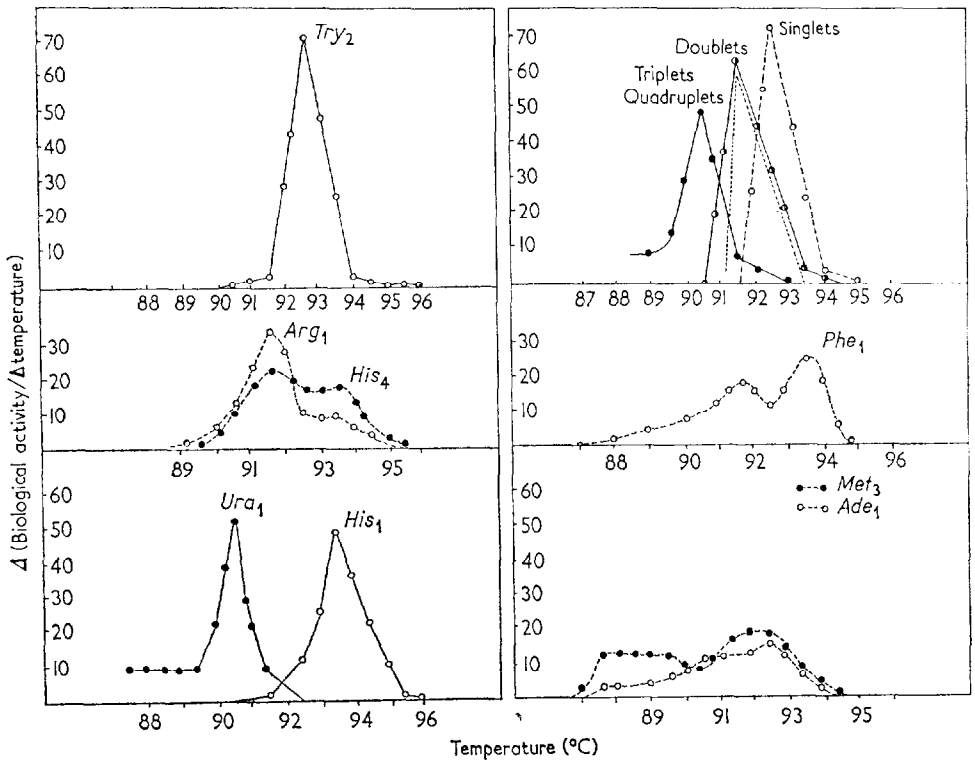
Thermal denaturation and marker inactivation

These experiments involve screening of the markers for their inactivation profile. The hyperchromicity as a function of temperature in standard saline citrate at pH 7·0 gives an inactivation midpoint (the midpoint of 90% of total increase) of 87·8 ± 0·5°C for *B. subtilis* DNA.

Denatured DNA samples showed varying biological activity, from 0 to 6%, due to differences in handling during the cooling procedure, although the density of such denatured DNA was always 1·718 to 1·720 g/cm³. In these experiments the activity of denatured DNA was zero or less than 0·01%. In cases where activity was observed, it was found on the lighter side of the distribution in the density-gradient.

Figures 6 and 7 present results for the thermal inactivation profiles of some markers; these are seen to correlate with their pycnography (Table 3). Marker inactivation can also be studied qualitatively by spot testing 10 μg control DNA and DNA heated to 91°, 93° and 95°C, respectively, on a plate containing competent cells carrying the marker in question (Plate I).

Plots of Δ (biological activity)/Δ (temperature) *versus* temperature are shown in



FIGS 8 and 9. Differential plots of (Δ in biological activity/Δ in temperature), against temperature, exhibiting the heterogeneity in the molecules of a given marker.

Figs 8 and 9. These differential plots are useful in displaying the heterogeneity of the molecules carrying a marker. For example, His_1 and Ura_1 are clearly separated and both have sharp single peaks. On the other hand, Phe_1, Ade_1 and Met_3 all show the same thermal resistance. His_4 shows two peaks of inactivation which are not readily noticed in the usual plots of activity against temperature. A possible explanation for the heterogeneity is that His_4 is located near a region rich in G–C, and during preparation of the DNA some segments carrying His_4 have broken off from this thermally stable region.

The inactivation profile of cotransfer of four linked markers, from single to quadruple cotransfer, was analysed. They all have the same melting point of 92·5°C when analysed singly. Thermal treatment rapidly destroyed the quadruplet cotransfer corresponding to a larger genetic region (whole molecules). Single markers were inactivated less rapidly. The following T_m(B) values for marker inactivation were obtained:

Cotransfer category	T_m(B)°C
Quadruplets	91·0
Triplets	91·4
Doublets	91·8
Singlets	92·5

The above results indicate that DNA molecules partly denatured by heat retain activity for some markers. Four of the histidine markers are thermally resistant, $T_m = 93{\cdot}8°C$, and His_2, situated between Try_1 and Try_2, shows a lower T_m.

Thermal denaturation and marker fractionation

In view of the possible correlation between thermal stability and density of some markers, a purification was attempted for the thermally resistant His_1 marker. Heating a DNA to 93·2°C spared the transforming activity of the few histidine genes but affected other markers to varying extents.

The distribution of both native and denatured fractions in the gradient is given in Fig. 10. Eighty to ninety per cent of the total input DNA is in the denatured density

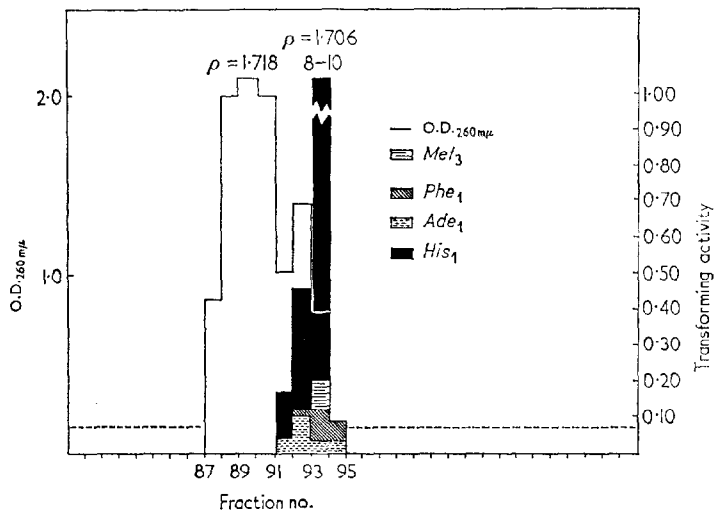


FIG. 10. 90 μg of DNA (heated at concentrations mentioned under Materials and Methods), was heated at 93·2°C in standard saline citrate and rapidly cooled. This was centrifuged in CsCl. Fractions were assayed for DNA concentration and biological activity as described under Materials and Methods. The density as measured in a CsCl density-gradient is given as ρ = g/cm³.

The dotted line represents the base line for O.D. at 260 mμ in the gradient other than the DNA distribution. The activities for Aro_2, Try_2, His_2, Try_1, Ura_1, Arg_7 and His_5 were less than 0·01%.

stratum. The resistant histidine markers are very little affected, but this DNA has no activity for Ura_1, Arg_7 and the entire linkage group. However, different amounts of Ade_1, Phe_1 and Met_3 activities were present, as the earlier observations on thermal inactivation midpoints had suggested. The transformation frequencies for determining the activity of different markers (Table 4) show that the specific activity of His_1 has increased tenfold.

Thermal renaturation

A DNA heated to 93·2°C and cooled rapidly by chilling, was centrifuged and the heat-resistant fraction was removed. The denatured DNA was renatured and assayed for biological activity. Table 4 shows the recovery of markers in the renatured DNA. His_1 has less than 8% activity, while the other markers had higher specific activities. The three intermediate markers are recovered with a lower efficiency, from 23 to 32% of the original activity.

TABLE 4

Specific activity of DNA heated to 93·2°C†

Marker	Percentage activity control native DNA	Percentage activity purified fraction	Percentage activity renatured DNA	Percentage activity denatured DNA
His$_1$‡	100	800–1000	8	0·01–0·001
Phe$_1$	100	18	37	0·01–0·001
Ade$_1$	100	11	29	0·01–0·001
Met$_3$	100	23	31	0·01–0·001
Try$_2$	100	0·001	48	0·01–0·001
Ura$_1$	100	0·001	51	0·01–0·001
Linkage group	100	-----	14	0

† DNA was heated at 93·2°C (as described in Materials and Methods), centrifuged and the native DNA fractions removed. The activity of this DNA for different markers is given under purified fraction. The denatured DNA fractions, from the same centrifugation experiment, were combined and renatured. The activity of this renatured DNA is given under renatured DNA. The denatured DNA represents a control sample which is completely denatured and not fractionated. Control DNA is the unheated native DNA. Its specific activity is set at 100% and the other values are related to this value.

‡ Also true of the *His*$_{12}$, *His*$_{13}$ and *His*$_{22}$.

TABLE 5

Properties of fraction of intermediate density

DNA samples	Transforming activity	Density (g/cm^3)
Control, native	1·0	1·703
Control, native and phosphodiesterase	0·87	
Intermediate fraction	0·13	1·713
Intermediate fraction and phosphodiesterase	0·02	

40 μg DNA heated in standard saline citrate at 93·2°C, rapidly cooled and centrifuged. Fractions in the strata of intermediate density were pooled and compared with the native fraction rich in *His*$_1$ marker activity. *E. coli* phosphodiesterase was added at a concentration of 5 units/ml. and the reaction was carried out for 30 min.

Partially denatured state of DNA

The pycnogram of DNA heated at 93·2°C suggested the presence of a minor partially denatured component represented by a significant left-skewness of the band at native density. The properties of this fraction are given in Table 5. A detailed analysis of such partially denatured molecules will be published elsewhere.

Physical properties of purified fractions

The thermally resistant molecules carrying the biological activity for *His*$_1$ were investigated in more detail. A purified fraction of the DNA recovered from centrifugation experiments was analysed for molecular weight, density, biological activity

and thermal denaturation profile. Besides, base composition was determined by paper chromatography of mononucleotides, originally labeled with ^{32}P in the DNA. Treatment with *E. coli* phosphodiesterase of the thermally resistant fraction recovered removed the contaminating denatured or partially denatured molecules, so that there was no skewness towards the heavier side of the gradient on recentrifugation. Figure 11 shows the distribution of this heat-resistant fraction. The molecular weight for this DNA is 16·7 million. The buoyant density is $1{\cdot}707 \pm 0{\cdot}001$ g/cm^3, which is at least 0·003 higher than the mean, equivalent to 3% more G–C pairs.

A heat-resistant fraction of DNA labeled with ^{32}P was analysed for the proportion of mononucleotides: this fraction assayed $48 \pm 2\%$ G–C. Spectral analysis of this fraction at 260 to 280 mμ also gives a ratio which suggests a low molar percentage of A–T compared to the native DNA (Table 6).

TABLE 6

Mononucleotide composition of DNA samples: ^{32}P cts/min

Mononucleotides	Control DNA	His_1 fraction
dAMP†	2280	2096
dTMP	2493	1994
dGMP	2010	2182
dCMP	1920	2208
Absorbance ratio 260/280	1·392–1·408	1·339

† Deoxynucleotides of the monophosphates of adenine, thymine, guanine and cytosine.

Other markers associated with the His_1 fraction are a few other histidine loci, His_5, His_{12}, His_{13} and His_{22}. Four out of five of the heat-resistant *His* can utilize histidinol for their growth (Table 7). Linkage between these markers has not been looked for. A fraction of native DNA carrying the maximum activity for transforming the linkage group, the purified His_1 fraction and the normal native DNA, were all centrifuged in the Spinco model E analytical centrifuge. At equilibrium, ultraviolet absorption photographs were taken. The densitometer tracings of these three DNA's, with their densities, are presented in Fig. 11.

TABLE 7

Growth pattern of some histidine mutants

Strain	Marker	T_m(B)§ (°C)	Growth with histidine	Growth with histidinol†	Period of growth (hr)‡
SB1	His_1	93·8	49	37	48
SB71	His_4	92·5	35	0	48
SB81	His_5	93·8	40	61	48
SB400	His_{22}	93·8	9	33	48
SB300	His_{13}	93·8	29	16	48

† Klett reading at 600 mμ.
‡ Biological activity.
§ T_m(B) is defined in legend to Table 3.

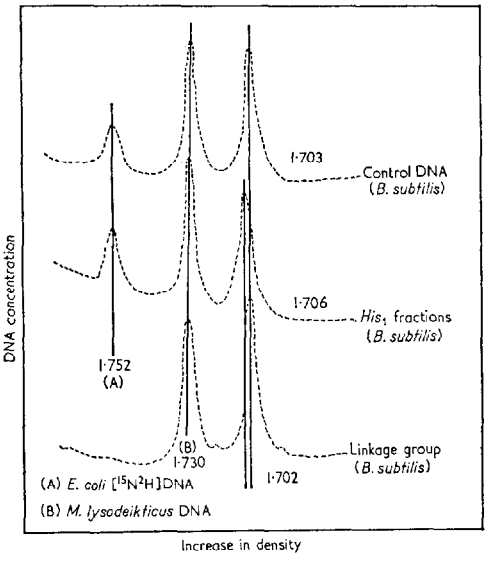


FIG. 11. Densitometer tracings of ultraviolet absorption photographs in model E analytical centrifuge. DNA samples, corresponding to purified His_1 fraction, fraction corresponding to highest activity for all the four markers in the linkage group and control were run with *E. coli* [$^{15}N^2H$]DNA (kindly given by Dr. C. Schildkraut) and *M. lysodeikticus* DNA as standards.

4. Discussion

The heterogeneity and distribution of DNA molecules from bacteria have been discussed by Sueoka (1961), and are also reflected in the nearest-neighbor frequencies for different DNA samples (Josse, Kaiser & Kornberg, 1961). The possible existence of heterogeneity and its effects on pycnography were pointed out by Baldwin (1959). Rolfe & Ephrussi-Taylor (1961) achieved partial separation of transforming activity for markers in pneumococcal DNA. Guild (1963), using pneumococcal DNA, found that density and thermal transition sequences changed significantly as a function of fragment size. In the present fractionation studies using *B. subtilis* DNA, the pattern of distribution is highly reproducible within these two DNA preparations of uniform size, but varies in others. The molecular weight of the purified DNA is probably the main factor in this non-reproducibility with different DNA samples. (A carefully prepared DNA preparation possessing an average molecular weight of 30 to 40×10^6 did not show the same pattern of separation of biological activity. Shearing the DNA gave marker separation of "His_1" markers. These results will be published elsewhere.) Apart from the observation on the denatured states of DNA, the work indicates significant fractionation with respect to some markers. This can be of the order of a three- to fourfold increase in specific activity for any marker as judged by "dose–response" relationships (Ganesan, 1963). The tenfold increase for His_1 markers could be achieved by partial denaturation followed by pycnographic separation. In the earlier investigations on denaturation with other systems, when the number of transformants was plotted against increasing temperature, there was always a significant increase in the specific activities for certain markers before the decrease due to collapse of the bihelix (Roger & Hotchkiss, 1961). *B. subtilis* DNA also exhibits

similar features, which may now be explained as due to the selective denaturation of certain molecules which compete during transformation. The ambiguities in G–C correlation and marker inactivation have been discussed by Geiduschek (1962). The His_1 marker partially purified here was also shown to be relatively more resistant than Try_2 to ultraviolet inactivation (Marmur *et al.*, 1961).

The principle results of this investigation can be summarized as the separation of *B. subtilis* DNA of molecular weight 11·6 million into fractions varying in chemical composition, melting point, buoyant density and specific genetic activity. The extreme fractions show a ratio of 10·5 : 1·0 for the activities of His_1 and Ura_1 markers. The ultimate aim is the 120-fold fractionation needed to separate the *B. subtilis* nucleus into its principal molecules of the size encountered in our preparations.

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